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# Monitoring Changes of Paramagnetically-Shifted $^{31}\text{P}$ Signals in Phospholipid Vesicles

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## **Abstract**

Phospholipid vesicles are commonly used as biomimetics in the investigation of the interaction of various species with cell membranes. In this paper we present a  $^{31}\text{P}$  NMR investigation of a simple vesicle system using a paramagnetic shift reagent to probe the inner and outer layers of the lipid bilayer. Time-dependent changes in the  $^{31}\text{P}$  NMR signal are observed, which differ whether the paramagnetic species is inside or outside the vesicle, and on the choice of buffer solution used. An interpretation of these results is given in terms of the interaction of the paramagnetic shift reagent with the lipids.

## **Introduction**

Biological cell membranes have an important dual role in that they must maintain the integrity of the cell contents, whilst simultaneously allowing required ions and molecules to be trafficked through channels and pores [1,2]. The membranes themselves are complex mixtures of various phospholipids and other molecules such as sterols (mainly cholesterol) [3], sphingolipids [2] and glycolipids [4]. In addition, numerous membrane proteins provide ion channels, pores and points of signal transduction across the membrane [2]. The exact composition of a cell membrane depends on both the role and location of the cell, and the organism in which the cell is found [2].

The complexity of cell membranes makes their investigation challenging, hence phospholipid vesicles are routinely used as mimics of biological cells [5]. The ability to prepare a vesicle comprising only key components of the membrane allows the role of individual parts to be investigated. Vesicles, and lipid bilayers, are also used to support membrane proteins allowing study in an environment enabling the adoption of their native folds [6-8]. This technique, and its variations, allows membrane proteins such as ion channels to be investigated [9]. In particular, the investigation of membrane proteins, supported in lipid bilayers and vesicles, by solid state nuclear magnetic resonance (NMR) spectroscopy has enabled structural studies to obtain levels of detail comparable to that seen with solution state NMR [6].

A number of biophysical techniques are routinely applied to the study of phospholipid vesicles, depending on the information required. For example, vesicle permeation studies typically employ a fluorescent dye which is contained in the centre of the



vesicle at self-quenching concentrations [10]. Any lysis of the vesicle causes release of the dye, a subsequent drop in dye concentration, and hence increase in fluorescence. This provides a robust and sensitive technique which has widespread applicability [10-12]. The use of  $^1\text{H}$  NMR spectroscopy to study membranes themselves is limited by the significant spectral overlap and general poor resolution of the spectra [13,14]. Recently, changes in  $^1\text{H}$  peak shape have been used to infer dynamic information on the interaction between various sterols and small lipid vesicles [14]. The use of other, often more dilute, NMR-active nuclei can alleviate this problem, depending on their location in or around the vesicle bilayer. For example, the functioning of ion channels, and their inhibition by various compounds such as toxins or anaesthetics has been investigated using  $^7\text{Li}$  and  $^{23}\text{Na}$  NMR and the use of paramagnetic shift reagents based on dysprosium (III) [15,16]. The lipids themselves, can be observed directly using the single phosphorus atom located in the head group of each molecule.  $^{31}\text{P}$  NMR spectroscopy is an ideal choice as it has reasonably high sensitivity with  $^{31}\text{P}$  being 100% abundant,  $I = \frac{1}{2}$  and relatively high receptivity [17]. There are numerous reports of the use of  $^{31}\text{P}$  NMR to investigate lipid bilayers and vesicles [18-21], with some evidence that the inner and outer layers can be distinguished by subtle differences in chemical shift, on the order of 0.1 ppm, arising due to differences in the curvature of the inner and outer layers [13,22]. As with the case of using alkali metals, this distinction of the layers, i.e. inside versus outside of the vesicles, can be enhanced by the use of paramagnetic shift reagents [23]. The interaction of a nuclear spin with unpaired electron spins causes a shift and/or broadening of a resonance [24]. This effect can be exploited by inclusion of a paramagnetic ion in the interior or exterior solvent volume. This results in a shift in the resonance position and a separation of signals from the inner and outer layers. The

choice of paramagnetic ion depends on a number of factors, such as the spin state of the metal centre and any potential interactions via the ligand framework [24].

Effective separation of the inner and outer layers has previously been achieved using neodymium (III) chloride [25], europium (III) chloride [25,26] or praseodymium (III) chloride [20,26,27]. Andrews et al. have shown that praseodymium (III) chloride induces a useful paramagnetic shift, comparable to europium (III), but with less line broadening than other lanthanides such as dysprosium (III) or terbium (III) [26]. Heteronuclear  $^1\text{H}$ - $^{31}\text{P}$  correlation experiments have also been reported, allowing the investigation of complex mixtures of lipids [28].

In this paper we present a temporal investigation of small POPC vesicles prepared by sonication using  $^{31}\text{P}$  NMR spectroscopy with  $\text{PrCl}_3$  as the paramagnetic shift reagent. The influence of the location of the paramagnetic species and the role of the buffer are investigated.

## **Materials and Methods**

### *Materials*

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and Tris (tris(hydroxymethyl)aminomethane) were obtained from Sigma Aldrich and used as received.

Vesicle samples were prepared by dissolving the required amount of lipid in a 2:1 chloroform / methanol (v/v) mixture. The samples were then evaporated to dryness under a stream of nitrogen and stored in a vacuum desiccator overnight. Lipid films

were then stored at -20 °C until required. The lipid films were resuspended in the appropriate solvent, either D<sub>2</sub>O or buffer and mixed by vortex to create a suspension. Vesicles were then prepared by placing the lipid suspension in a round-bottomed flask and sonicating at 35 °C in a bath sonicator for approximately 3 hours or until the solution became clear [10,29].

Praseodymium (III) chloride hydrate was purchased from Sigma Aldrich (Dorset, UK) in a sealed ampule and used as obtained. For samples with praseodymium (III) on the outside of the vesicles, aliquots of appropriate stock PrCl<sub>3</sub> solutions (30 mM), prepared in the corresponding buffer, were added after sonication, whilst when incorporating Pr<sup>3+</sup> inside the vesicles PrCl<sub>3</sub> was added to the resuspension buffer at the required concentration. For these samples the vesicles were eluted through a Sephadex G-50 minicolumn following sonication in order to perform a buffer exchange and remove the praseodymium (III) chloride from the exterior solution, whilst retaining the encapsulated PrCl<sub>3</sub> in the vesicle inner volume.

#### *Nuclear Magnetic Resonance Spectroscopy*

NMR data was collected on a Varian VNMRs 600 spectroscopy (Agilent Technologies, Yarnton, UK) using a 5 mm X{<sup>1</sup>H} room temperature probe equipped with an actively-shielded z-gradient coil. The sample temperature was regulated at 35 °C. The <sup>31</sup>P 90° pulse width was 12.5 μs and <sup>1</sup>H decoupling was provided using a 3.5 kHz WALTZ16 modulated B<sub>2</sub> field. Spectra were recorded using 32k complex points over a spectral width of 14.7 kHz. NMR data were processed with 100 Hz exponential line broadening prior to Fourier transformation using the Mnova suite from Mestrelab Research (Santiago de Compostela, Spain). Peak areas were obtained by fitting

Lorentzian lineshape(s), with the signal intensity given by the product of the fitted height(s) and width(s).

#### *Dynamic Light Scattering*

DLS measurements were performed using a Malvern Zetasizer Nano-S (Malvern Instruments, Worcestershire, UK). Samples were diluted to a concentration of 1 mg mL<sup>-1</sup>, using the appropriate buffer solution. The data were analysed using the Dispersion Technology Software supplied with the instrument.

### **Results and Discussion**

#### *Vesicle Preparation*

Phospholipid vesicles of 1-palmitoyl-2-oleoyl-sn-3-phosphocholine (POPC) were prepared by sonication in a buffer solution containing 10 mM HEPES and 100 mM sodium chloride. The trace in Figure 1(a) shows the <sup>31</sup>P NMR spectrum of the vesicles, along with a diagram of a unilamellar vesicle. A single broad resonance is observed at approximately -1 ppm for the phosphate head group. This is consistent with the formation of large multiunit assemblies in solution [18-21]. The previously reported difference in chemical shift for the inner and outer layer of the vesicle [20] is not seen in these spectra due to the larger spectral line width arising from an increased relaxation due to chemical shift anisotropy and working at higher static magnetic field [30]. Addition of praseodymium (III) chloride to the outer solution after sonication results in the spectrum in Figure 1(b). There are now clearly two distinct resonances, with the signals from the outer layer shifted to higher chemical shift, approximately 8 ppm, by the paramagnetic Pr<sup>3+</sup> ions [18]. The paramagnetic shift induced by praseodymium (III) ions is principally dipolar in origin [24,31]. The vesicles

themselves have a diameter of around 16-20 nm as measured using DLS, shown as the traces in Figure 1(c). A very small second component is also seen in the traces, corresponding to vesicles with a diameter of around 200 nm. This component is not observable in the  $^{31}\text{P}$  NMR spectra due to efficient spin-spin relaxation as the larger vesicles relax more rapidly by virtue of a long rotation correlation time leading to significant line broadening [30].

The relative area of the two signals for the inner and outer layers of bilayer ( $\sim 0.6$ ), combined with the DLS measurement of overall diameter allows the thickness of the lipid bilayer to be determined, assuming the lipids are organised over the surface of a sphere. For the vesicles produced and used here, this analysis yields a bilayer thickness of approximately 3.7 nm. This is consistent with a phosphate spacing of 3.8 nm across the bilayer reported for POPC vesicles using X-ray scattering data [32].

### *Stability Time-series*

In order to monitor the stability of a fresh vesicle solution,  $^{31}\text{P}$  spectra were acquired every hour for a period of 68 hours with  $\text{PrCl}_3$  outside the vesicle. The results are shown in Figure 2(a). Over the course of the 68 hours, the signal corresponding to the inner layer of the vesicle was observed at a constant chemical shift of -1 ppm. There is some minor variation in the height of the signal, but the overall signal intensity (i.e. height  $\times$  width) remained constant. What is clearly noticeable, however, is that the resonance for the exterior layer moved to higher chemical shift, from 8 ppm to 12 ppm, over the course of 68 hours, with a concomitant decrease in signal height. Interestingly, the overall signal intensity remains constant. Figure 2(b) shows these

data plotted as a function of time. The change in chemical shift can be fitted using a single exponential function of the form:

$$\delta(t) = \delta^{\infty} - A\exp(-t/\tau) \quad (1)$$

The results of fitting this equation to the data given in Figure 2(b) results in the parameters quoted in Table 1. This shows that the  $^{31}\text{P}$  signal changes position with a time constant of around 27 hours. The high  $R^2$  value indicates a good fit to the experimental data.

Figure 2(c) shows the peak areas plotted as a function of time. As noted above, there is no significant change in the areas of either the interior or exterior peak over the course of the experiment. The lower trace shows the ratio of the peak areas, which remains constant, with an average value of 0.6. This indicates that the vesicles remain intact over the course of the experiment, with little or no change in their overall composition or size. The only change is in the nature of the interaction between the paramagnetic  $\text{Pr}^{3+}$  and the outer lipid layer. These results are very reproducible with vesicle preparations from different batches of lipid showing very similar results.

The effect of the concentration of the paramagnetic shift reagent was investigated by doubling the amount of  $\text{PrCl}_3$  used in the exterior buffer solution. Since the paramagnetic shift is mainly dipolar in origin, the use of an increased amount of shift reagent should result in a greater paramagnetic shift [24], which was observed with these vesicles. The overall temporal behaviour of the system is broadly similar, with an increase in  $^{31}\text{P}$  chemical shift from an initial value of 9 ppm to approximately 14.5 ppm observed over the same duration of the experiment. This data is also plotted in Figure 2(b). Fitting equation 1 to these data revealed a similar time constant for the

process (see Table 1) to that obtained in the case of 3 mM praseodymium (III) chloride, suggesting that the rate of peak shift is not dependent on the concentration of the paramagnetic species.

### *Buffer Choice*

The change in the vesicle  $^{31}\text{P}$  spectrum over time indicates some variation in the nature of the interaction of the paramagnetic praseodymium (III) species with the vesicle during the experiment. In order to determine whether the HEPES buffer was responsible for the observed downfield shift in the exterior  $^{31}\text{P}$  signal, a change in buffer to Tris, another common buffer system used in biological experiments, was performed. This resulted in similar behaviour being observed: the signal for the outer lipid layer moved approximately +4.5 ppm to higher chemical shift over the duration of the experiment. The time series, and analysis using Equation 1, is shown in Figure 3(a). The time constant for the process is comparable to that observed when using HEPES buffer. The variation in peak area over the time series is plotted in Figure 3(b), showing that there is little change. The area ratio is constant at around 0.6. As before, this indicates that the vesicles are stable over the time period of the experiment, and suggests that it is only the interaction between the  $\text{Pr}^{3+}$  ions and the vesicle which is changing.

HEPES and Tris have the potential to bind metal ions. Tris is well known to act as a chelating agent, while HEPES was originally described as a non-chelator [33], more recently it has been shown to bind “soft” metals such as silver (I) [34], mercury (II) [35] and ruthenium (II) [36]. To test whether the buffers were interacting with the praseodymium (III), POPC vesicles were prepared in sodium chloride solution only,

with 3 mM  $\text{PrCl}_3$  added to the exterior of the vesicle as before. The corresponding  $^{31}\text{P}$  spectra are shown in Figure 4(a). A similar splitting of the interior and exterior signals was seen, however, there was little change in the signals' position, width or height over the 64 hour time series. The interior signal remained at -1 ppm, while the exterior resonance moved only slightly, from around 8.3 ppm to approximately 9 ppm. These results, shown in Figure 4(b), are similar to the chemical shift observed at the  $t = 0$  point in the case of the buffer system containing HEPES or Tris. This therefore suggests that the change in chemical shift of the exterior lipid signal over the time period of around 70 hours, is the result of some interaction between the  $\text{Pr}^{3+}$  ions, HEPES or Tris buffer and the phosphate head groups of the lipids. There are reports of the interaction of various lanthanides with the phosphate head group [18,37,38], however, investigation of the role of the buffer appears to be neglected.

#### *Paramagnetic Shift Reagent on the Vesicle Interior*

Many experiments into vesicle permeation utilise a reporter encapsulated inside the vesicle, such as a fluorescent dye, which is then released upon membrane permeation [10]. With this in mind, a second series of vesicles were prepared in which 6 mM praseodymium (III) chloride was encapsulated inside the vesicles, with the corresponding buffer solution on the outside. As before, two sets of signals were observed in the  $^{31}\text{P}$  NMR spectrum, shown in Figure 5(a), this time a sharp resonance at -1 ppm corresponding to the exterior layer of lipid and a second resonance at around 11 ppm for the inner layer. This inner layer signal was found typically to be broader than that observed for the exterior peak in the case when the paramagnetic shift reagent is on the outside of the vesicle (see above). This may be due in part to



the increased curvature of the inner layer [13,22] and the greater contact between the paramagnetic species and the inner phospholipid layer.

The  $^{31}\text{P}$  NMR spectrum of the vesicles with  $\text{Pr}^{3+}$  encapsulated inside was monitored every 30 minutes over a period of 66 hours. The data is presented in Figure 5(a). In contrast to the data shown above, this time, the paramagnetically shifted signal moved to lower chemical shift over the course of the experiment, moving from around 11 ppm to approximately 5 ppm. The change in chemical shift can be fitted to an exponential function (equation 1), as shown in Figure 5(b), yielding the parameters quoted in Table 1. The time constant for this process is markedly smaller than that observed for the  $\text{PrCl}_3$  on the exterior of the vesicle, being around 3.66 hours. It is also worth noting that the peak areas remain constant over the time course of the experiment, shown in Figure 5(c). The ratio of the peak areas remains constant at around 0.5. This is smaller than that observed when the praseodymium (III) is present on the outside of the vesicles. The reasons for this smaller ratio are not clear, but may be related to the interaction of the paramagnet with the vesicle inner surface.

#### *Shift Reagent Inside and Out*

As a final control experiment, 6 mM praseodymium (III) chloride was both encapsulated within the vesicles, and present in the exterior solution. In this case, both signals for the inner and outer layers are shifted by the dipolar interaction with the paramagnetic  $\text{Pr}^{3+}$  centre. The resulting spectra, recorded every 30 minutes over 88 hours, are shown in Figure 6. A broad signal, with a shoulder to the higher chemical shift side, is seen, which does not appear to change over time. Fitting a pair of Lorentzians to the spectrum allows identification of two contributing signals, which

can be tentatively assigned to the inner and outer layers, based primarily on the relative intensity of the two components. The ratio of the two peak areas is 0.5, which is similar to that observed when  $\text{PrCl}_3$  is only present in the interior of the vesicle.

There are a number of NMR-based techniques available to probe the interaction of small molecules with large biomolecular species, typically proteins [39]. These experiments rely on selective excitation of  $^1\text{H}$  resonances from the large biomolecular species, and subsequent dipolar driven transfer, i.e. Overhauser effects, to the small molecule binder for detection [40]. The idea would be to look for an interaction between the lipid vesicle and the potential buffer / praseodymium (III) complex. In their current form these experiments are, unfortunately, impractical for the systems described in this article since the required  $^1\text{H}$  NMR signals for the buffer compounds overlap with the alkyl signals of the phospholipids. It may be possible to develop suitable heteronuclear versions of these experiments, similar to those for probing the interaction of fluorinated drug molecules with protein targets [41,42].

The dynamics of phospholipid vesicles are ideally suited to study by NMR spectroscopy. Changes in  $^1\text{H}$  line shape as a function sterol concentration have been used to probe mobility of the lipid molecules [14] and atomic resolution molecular dynamics simulations have been used to probe the nature of the lipid water interface [43]. One of the most powerful methods is the use of  $^2\text{H}$  NMR to provide information on the dynamics and alignment of the lipids within the vesicles, for example in monitoring the fusion of small and giant unilamellar vesicles [44]. Similar experiments have also been used to probe the interaction of lipid membranes with polyelectrolytes [45]. It is possible that experiments similar to these could shed light

on the effects described here and determine if reorganisation of the lipid bilayer is occurring upon addition of the paramagnetic praseodymium (III) chloride.

## **Conclusions**

Phospholipid vesicles are used as model systems for a wide range of investigations, including vesicle permeation by amyloidogenic peptides, [10,46] changes in membrane dynamics upon protein binding [22] and the transport of ions and small molecules across a membrane [15,16]. The vesicles are typically prepared by simple methods, such as sonication or extrusion, and often stated as being “stable over the duration of the experiment”. In this work we have shown that while small, simple POPC lipid vesicles are stable over periods of between 24-90 hours as determined by the constant peak areas for the interior and exterior  $^{31}\text{P}$  NMR signals, there are, however, some changes which occur over this time scale, notably changes in the chemical shift of the paramagnetically shifted  $^{31}\text{P}$  resonance. These changes are dependent on the nature of the buffer used in the sample preparation and on the location of the paramagnetic species, whether it is located in the vesicle interior or in the exterior solution. The results suggest that there is some maturation or ripening process occurring between the  $\text{Pr}^{3+}$  ions, the buffer and the lipid bilayer, the exact nature of which is not clear at this stage, however, could be related to a partial equilibration of the lipid vesicles. Once this event is complete, no further change in the  $^{31}\text{P}$  NMR spectrum is observed.

The results presented here indicate that using phospholipid vesicles as a mimetic of membrane interaction care should be taken using paramagnetic shift reagents and freshly prepared vesicles that any changes in  $^{31}\text{P}$  peak position or appearance over the

early time points of an experiment is not simply a result of this “maturation” or re-equilibration process, but arises from the action under investigation. Therefore we suggest that suitable control experiments are performed along side to allow any complicating factors to be eliminated.

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## Tables

*Table 1:* Parameters returned from fitting Eq. 1 to the various  $^{31}\text{P}$  time course experiments described in the main text.

| Sample                           | Buffer | $\delta^\infty$ / ppm | $A$ / ppm | $\tau$ / hours | $R^2$ |
|----------------------------------|--------|-----------------------|-----------|----------------|-------|
| 3 mM $\text{Pr}^{3+}$ , exterior | HEPES  | 12.465                | 4.811     | $27.6 \pm 1.4$ | 0.995 |
| 6 mM $\text{Pr}^{3+}$ , exterior | HEPES  | 14.643                | 5.388     | $25.3 \pm 1.3$ | 0.991 |
| 3 mM $\text{Pr}^{3+}$ , exterior | Tris   | 13.550                | 4.902     | $25.4 \pm 1.3$ | 0.996 |
| 6 mM $\text{Pr}^{3+}$ , interior | HEPES  | 4.859                 | -7.883    | $3.06 \pm 0.2$ | 0.969 |

## Figure Captions

*Figure 1:* Proton-decoupled  $^{31}\text{P}$  spectra of POPC vesicles (a) without and (b) with 3 mM praseodymium (III) chloride in added to the buffer solution. The spectra were acquired under identical conditions. (c) Shows DLS traces for the vesicle sample, as used in (a), measured in triplicate.

*Figure 2:* (a) Proton-decoupled  $^{31}\text{P}$  NMR spectra of POPC vesicles, with 3 mM  $\text{PrCl}_3$  in the exterior HEPES solution recorded over a period of ~68 hours. Every second spectrum is shown. (b)  $^{31}\text{P}$  chemical shifts as a function of time for the exterior and interior phosphorus signals for both 3 mM and 6 mM  $\text{PrCl}_3$  systems. (c) Peak areas, and peak-area ratio, for POPC vesicle with 3 mM  $\text{PrCl}_3$  in the exterior solution.

*Figure 3:* (a)  $^{31}\text{P}$  chemical shifts as a function of time for the exterior and interior phosphorus signals for 3 mM  $\text{PrCl}_3$  in the exterior solution using Tris buffer. (b) Peak areas, and peak-area ratio, for the sample in (a).

*Figure 4:* (a) Proton-decoupled  $^{31}\text{P}$  NMR spectra of POPC vesicles, with 3 mM  $\text{PrCl}_3$  in the exterior solution, without any buffer, recorded over a period of ~68 hours. Every second spectrum is shown. (b)  $^{31}\text{P}$  chemical shifts as a function of time for the exterior and interior phosphorus signals.

*Figure 5:* (a) Proton-decoupled  $^{31}\text{P}$  NMR spectra of POPC vesicles, with 3 mM  $\text{PrCl}_3$  in the interior solution, recorded over a period of ~68 hours. Every second spectrum is shown. (b)  $^{31}\text{P}$  chemical shifts as a function of time for the exterior and interior phosphorus signals. (c) Peak areas, and peak-area ratio, for POPC vesicle with 3 mM  $\text{PrCl}_3$  in the interior solution.

*Figure 6:* Proton-decoupled  $^{31}\text{P}$  NMR spectra of POPC vesicles, with 6 mM  $\text{PrCl}_3$  in both the exterior and interior solutions, recorded over a period of ~88 hours. Every second spectrum is shown.













